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## DRY ANALYTICAL ELEMENT FOR ACETAMINOPHEN ASSAY

5 INS } This is a continuation-in-part of application Serial No. 014,915, filed July 15, 1992, now US Patent 6,015,687

### FIELD OF INVENTION

10 This invention relates to a spectrophotometric assay for the detection of acetaminophen in aqueous fluids which is carried out with a dry analytical element..

### BACKGROUND OF THE INVENTION

15 Acetaminophen is a widely used analgesic. It is available without a prescription and is often used when aspirin may present problems to a patient. At therapeutic doses, serum concentration is usually below 50 mg/L. Toxicity is usually observed if the serum concentration four hours after  
20 ingestion of the drug is greater than 300 mg/L. One effect of overdose is liver toxicity. The need for an accurate method of determining the concentration of acetaminophen in serum is therefore apparent.

Known methods of assaying anilides such as acetaminophen utilize  
25 arylacylamidase (E.C.3.5.1.13) and an oxidizing agent. Arylacylamidase cleaves the amide bond of the anilide to produce acetate and an aniline such as p-aminophenol. The aniline is then made to react with a color-forming compound like phenol in the presence of an oxidizing agent such as permanganate or the metal salts of copper or iron to form a color compound  
30 like indophenol which can be detected at 615 nm. Other methods use oxidizing agents like periodate or persulfates. US Patents 4,999,288 and 4,430,433 are typical.

One disadvantage of these methods is that most metal salts oxidize the  
35 aniline very slowly unless they are at alkaline pH, a condition that inactivates arylacylamidase.

US Patent 4,675,290 (issued June 23, 1987, to Matsumoto) discloses a method of assaying peptidase to enzyme activity which comprises treating a

synthetic dibrominated amide (as substrate) with a sample containing  
peptidase thereby liberating a dibrominated aniline; oxidizing the thus-  
liberated dibrominated aniline with an oxidase (e.g. ascorbate oxidase) which  
consumes oxygen and forms pigment by oxidative condensation of said  
5 aniline in the presence of a coupler of a defined formula. This reaction takes  
place in solution.

Matsumoto's method uses an enzyme and might therefore overcome the  
problems encountered with the inorganic oxidizing agents of the prior art.  
10 However, Matsumoto employs a synthetic dibrominated substrate and is  
therefore not directly applicable to a test for acetaminophen. There is  
evidence in the art that in the case of oxidative coupling of phenols for color  
formation, color yield can be increased when certain halogenophenols are  
used in the color-forming reaction. However acetaminophen as it appears in  
15 biological fluids to be tested is not halogenated and is therefore chemically  
distinguishable from the dibrominated synthetic substrate used in  
Matsumoto's method.

Further, Matsumoto disclosed a solution assay. An alternative and more  
20 convenient assay involves "dry" chemistry, a term that refers to methods and  
techniques that are carried out using chemical reagents contained in various  
"dry-to-the-touch" test elements such as "dip and read" test strips, multilayer  
test elements and the like. "Dry" methods require no liquid for reconstitution  
or analysis other than the test sample.

25 Reagents employed in solution assays often do not perform well when  
adapted to a dry format. Dry elements utilize minute amounts of reagents and  
test samples and so in a dry element for acetaminophen assay, it is important  
to employ reagents that give a clear and precise color signal when aniline is  
30 oxidatively coupled to a coupler to provide color.

The range of compounds that could be chosen as potential couplers is  
very wide. Matsumoto neither suggests nor gives guidance to those color  
forming oxidative couplers that would perform well in dry chemistry. The  
35 problem is that the selection of a suitable oxidative coupler for a dry element  
is essentially empirical.

It would be desirable to have a dry assay for determining acetaminophen  
in biological fluids comprising enzymes and other reagents which remain

active when combined and coated in dry format and which give a clear and precise signal on oxidative coupling with a suitable coupler.

### SUMMARY OF THE INVENTION

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The present invention selects for use a coupling agent that provides a detectable color change and precise determination of acetaminophen in a dry format. It was discovered that a water-soluble coupling agent was required and that a water-insoluble coupling agent, even if appropriately dispersed, did not work well.

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The element of the present invention avoids the inorganic oxidizing agents taught by the prior art and uses instead mild oxidizing agents, such as an enzyme or a ferricyanide. In dry analytical elements, oxidizing agents like  $\text{Cu}^{++}$  and most  $\text{Fe}^{+++}$  salts are known to attack a gelatin matrix by crosslinking and thus hardening the gelatin. The use of an enzyme as an oxidizing agent overcomes this problem. Suitable oxidizing enzymes include ascorbic acid oxidase, lactase and tyrosinase.

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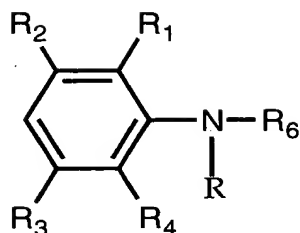
The ferricyanide is not as strong an oxidizing agent as most  $\text{Fe}^{+++}$  salts or other metal oxidizing agents (see Table A). It is unexpected, therefore, that a ferricyanide would be able to provide fast reaction kinetics at neutral pHs without it damaging the dry element structure. Suitable ferricyanides include the alkali metal and alkaline earth metal ferricyanides such as, for example, potassium, sodium and calcium ferricyanide. The preferred ferricyanides are the alkali metal ferricyanides.

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Accordingly, there is provided an analytical element for the determination of acetaminophen in an aqueous fluid comprising a support having thereon at least one reagent layer and containing in said reagent layer:

- (a) an arylacylamidase enzyme;
- (b) an oxidizing enzyme or a ferricyanide capable of oxidatively coupling paraaminophenol to a coupling agent to form a color compound.
- 35 (c) a water-soluble, color-forming, coupling agent of the general structure:



wherein R is a water-solubilizing group selected from  $-(CH_2)_nX$  where n is 1 to 5 and X is  $-SO_3M$  where M is hydrogen, an alkali metal, an alkaline earth metal or an ammonium ( $NH_4^+$ ) cation, or  $-N(R_7)_3^+Z^-$  where each R<sub>7</sub> is independently selected from alkyl of 1 to 4 carbon atoms such as methyl, ethyl, propyl and butyl, and Z is an acid anion such as chloride, bromide, iodide, fluoride, p-toluenesulfonate, and the like, or X is  $(-OCH_2CH_2)_yOH$  where y is 2 to 5;

R<sub>1</sub> and R<sub>6</sub> are taken together to represent an ethylene, trimethylene, or tetramethylene group which forms a partially saturated ring;

R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub> are independently selected from hydrogen, alkyl of 1 to 4 carbon atoms, and alkoxy of 1 to 4 carbon atoms.

Another embodiment of the invention provides a method for determining acetaminophen in an aqueous liquid comprising the steps of:

- a. contacting a sample of the aqueous liquid with the analytical element described above; and
- b. correlating the amount of color compound formed to the concentration of acetaminophen in the fluid.

An advantage of the invention is that the water-soluble coupling agent and either the enzyme-catalyzed oxidative coupling or the ferricyanide coupling allow for a rapid reaction. We were able to develop a detectable color signal in 57 seconds. Water-insoluble coupling agents involve much slower reactions.

It was surprising to find that in order to obtain an acceptable amount of color product in the desired time (5 minutes), it was necessary to combine a water-soluble coupling agent and an enzyme. Even in solution assays, in

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- order to obtain results in 5 minutes, the oxidizing enzyme (e.g. ascorbate oxidase) had to be premixed with a water-soluble coupling agent prior to dilution with buffer. The reason for this is not known. While not wishing to be bound by scientific theories, Applicants believe that water solubility of the
- 5 coupling agent allows it to mix sufficiently with the enzyme in the analytical element during storage (i.e. between manufacture and use). It appears that prior contact between the coupling agent and the oxidizing enzyme before reaction is necessary to obtain a satisfactory color reaction in 5 minutes.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the performance of an analytical element of the invention when 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline (compound #2, THQSO<sub>3</sub>H) is used as the coupling agent.

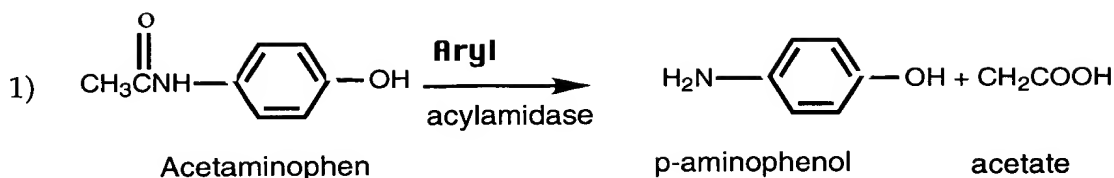
Figure 2 is a graph showing the performance of a comparative element using 1-(n-butyl)-1,2,3,4-tetrahydroquinoline (compound #1, BTHQ) as the coupling agent.

Figure 3 is a graph showing the performance of another comparative element using 1,2,3,5,6,7-hexahydrobenzo[ij]quinolizine, (compound #4, Julolidine) as the coupling agent.

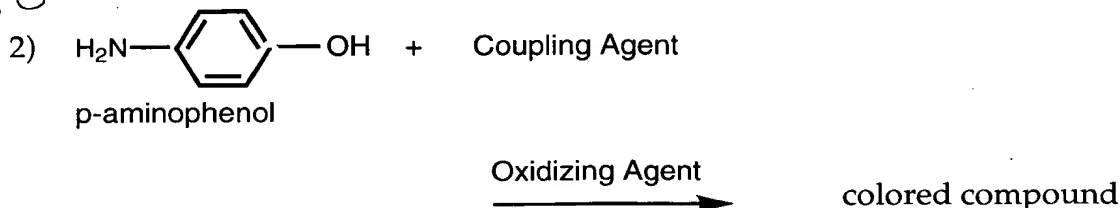
Figure 4 is a graph showing the performance of an analytical element of the invention using 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline (compound #2, THQSO<sub>3</sub>H) as the coupling agent and using potassium ferricyanide as the oxidizing agent.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses a colorimetric assay for the determination of acetaminophen based upon the enzymatic hydrolysis of acetaminophen and the subsequent detection of a metabolite of the hydrolysis. More specifically, the assay uses a first enzyme, arylacylamidase (E.C.3.5.1.13), to cleave the amide bond of acetaminophen to produce acetic acid and p-aminophenol. A second enzyme such as ascorbic acid oxidase (E.C.1.10.3.3), tyrosinase (E.C.1.14.18.1), or lactase (E.C.1.10.3.2) oxidizes the p-aminophenol so that it couples to a water-soluble coupling agent as defined herein, to form a dye. Ferricyanide can also be used to oxidize the p-aminophenol. The dye is detectable at 670 nm. The reaction proceeds as follows:



T<sub>10080</sub>



The water-soluble coupling agents useful in the present invention were identified by screening and selecting from among several various couplers of similar structure. Those that were insoluble did not provide enough coupling (dye formation) under the test conditions to produce enough absorbance for analytical discrimination of a wide dynamic range of analyte concentrations. Those of the structure described hereinbefore and wherein R<sub>1</sub> and R<sub>6</sub> are taken together to represent trimethylene, i.e., to complete a partially saturated 6-membered, nitrogen-containing heterocyclic ring, have particularly desirable absorption maxima (about 670 nm) and are especially useful in analytical elements because the absorption is not masked by interferents, such as bilirubin, which absorb at lower wavelengths. The preferred water-soluble species is 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline. The screening procedures and their results are described hereinafter. The amount of color compound formed at the end of the reaction indicates the concentration of acetaminophen in the test sample.

The above-described reagents are coated on a support to provide a dry analytical element of the invention. elements can be configured in a variety of forms, including elongated tapes of any desired width, sheets, slides, or chips.

The elements can be used in manual or automated assay techniques. In general, in using the elements, acetaminophen determination is made by taking the element from a supply roll, chip packet, or other source and physically contacting it with a sample (for example, up to 200 mL) of the liquid to be tested so that the sample and reagents interact sequentially within the element and become mixed. Such contact can be accomplished in any suitable manner, for example, by dipping or immersing the element into the

sample or, preferably, by spotting the element by hand or machine with a drop of the sample with a suitable dispensing means.

After sample application, the element is incubated, for a period of up to 5 minutes, to facilitate color development. By incubation it is meant that the reagents are maintained at 37°C in contact with each other for a period of up to 5 minutes before color measurements are made. Aqueous liquids which can be tested in the assay include, but are not limited to, whole blood, plasma, sera, lymph, bile, urine, spinal fluid, sputum, perspiration and the like as well as stool secretions. It is also possible to assay fluid preparations of human or animal tissue such as skeletal muscle, heart, kidney, lungs, brains, bone marrow, skin, and the like.

Dry analytical elements useful for the assay of liquids can be prepared according to the teachings of US 3,992,158 and US 4,357,363, the contents of which are incorporated herein in their entirety.

Briefly described, the analytical element of this invention comprises one or more layers coated on a suitable support. All the reagents may be in a single layer coated on the support. Preferably, the reagents are coated in two distinct reagent layers as shown in Table I below. Whether contained in the same or in different layers of the element, all reagents must be in fluid contact with each other, meaning that reagents and reaction products can pass within a layer and between superposed regions of adjacent layers. In other words, when the element is contacted with an aqueous fluid, all reagents of the analytical composition of this invention are mixed sequentially as stated hereinbefore.

The support can be any suitable dimensionally stable, and preferably, nonporous and transparent (i.e. radiation transmissive) material which transmits electromagnetic radiation of a wavelength between about 200 and about 900 nm. A radiation-transmissive support is particularly preferred to enhance and facilitate determination of detectable changes occurring in these elements by use of various radiation detection methods. A support of choice for a particular element should be compatible with the intended mode of detection (reflection, transmission or fluorescence spectroscopy). Useful support materials include polystyrene, polyesters (e.g. poly(ethylene terephthalate)), polycarbonates, cellulose esters (e.g., cellulose acetate), etc.



At least one reagent layer is coated on the support. The reagent layer(s) contain the indicator composition comprising one or more reagents dispersed in one or more synthetic or natural binder materials, such as gelatin or other naturally-occurring colloids, as well as different synthetic hydrophilic polymers such as polyacrylamide, poly(N-vinyl-2-pyrrolidone), poly(acrylamide-co-N-vinyl-2-pyrrolidone), copolymers of the above, and polymers or copolymers to which crosslinkable monomers have been added.

The reagent layer may contain a buffer. Useful buffers include phosphate, pyrophosphate, tris(hydroxymethyl)aminomethane (TRIS), 2[[tris(hydroxymethyl)methyl]amino]-1-ethanesulfonic acid (TES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[4-(2-hydroxyethyl)-1-piperazinyl]propanesulfonic acid (EPPES), 2-hydroxy-3-[N-tris(hydroxymethyl)methyl]amino]-propanesulfonic acid (TAPSO), and other buffers with pH in the range of 6.5 to 8.5, preferably 7.5. The buffer may be in any or all layers of the element, or it may be in a separate layer devoid of other reagents.

Several surfactants such as Olin-10G<sup>TM</sup>, TX<sup>TM</sup>-102, TX-405<sup>TM</sup>, Zonyl FSNT<sup>TM</sup>, but preferably TX-100<sup>TM</sup>, and TX-165<sup>TM</sup> (the TX surfactants being a family of octylphenoxy polyethoxy ethanol nonionic surfactants sold by Union Carbide) may optionally be included in the reagent layer. Several different cross-linking agents are also optional, such as bisvinylsulfonylmethane, gluteraldehyde, etc.

The element may be provided with a porous, reflective spreading layer to uniformly distribute the liquid test sample over the element. The spreading layer may contain reagents, but preferably it is a distinct layer as shown in Table I below. Materials for use in spreading layers are well known in the art of making dry analytical elements as disclosed, for example, in US 4,258,001 and the above cited patents.

Useful spreading layers can be prepared using fibrous materials either mixed with a suitable binder material or woven into a fabric, as described in US 4,292,272 (issued September 29, 1981 to Kitajima et al), polymeric compositions or particulate materials, for example, a blush polymer such as disclosed in US 3,992,158, beads bound together with or without binding adhesives, as described in US 4,258,001 (issued March 24, 1981 to Pierce et al), and US 4,430,436 (issued February 7, 1984 to Koyama et al), and Japanese Patent Publication 57(1982)-101760. Particularly useful spreading

layers comprise barium sulfate or titanium dioxide. Since the sample is generally applied directly to the spreading layer, it is desirable that the spreading layer be isotropically porous, meaning that the porosity is the same in each direction in the layer as caused by interconnected spaces or pores between particles, fibers, or polymeric strands. An exemplary spreading layer is presented in Table I.

In one embodiment, the spreading layer contains N-ethylmaleimide, a compound found to be useful in blocking certain interferents which may be present in the test fluid. Commonly owned US application by T. C. Arter et al titled *Sulfhydryl Complexing Agents in Clinical Test Elements* discloses this invention and is filed on even date herewith.

Other optional layers, e.g. subbing layers, radiation-blocking layers, etc, can be included if desired. The layers of the element can contain a variety of other desirable but optional components, including surfactants, thickeners, buffers, hardeners, bacteriostats, antioxidants, coupler solvents, and other materials known in the art. The amounts of these components are also within the skill of a worker in the art.

Changes in the element can be detected with suitable spectrophotometric apparatus, usually a reflectometer, using generally known procedures disclosed, for example, in US 3,992,158 at Cols. 14-15, and US 4,357,363 at Col. 27. In an enzymatic reaction, the resulting product is determined by measuring, for example, the rate of change of reflection or transmission density in a finite area of the element of the invention contacted with the assay sample. The area measured is generally from about 3 to about 5 mm.

Representative elements of this invention are presented in Tables Ia and Ib below.

//

Table Ia

Acetaminophen Element Using Oxidase Enzyme

Coverage, g/m<sup>2</sup>\*

<u>Reagents/Spreading Layer</u>	<u>Useful Range</u>	<u>Actual Example</u>
Barium Sulfate	75-120	100
Cellulose	1-20	8
Surfactant TX-405 <sup>TM</sup>	0.2-4	1.6
Estane	1-15	12.3
Maleimide	0.1-3.0	1.0

Subbing Layer

Polyvinylpyrrolidone	0.5-2.5	1.0
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Reagent Layer 1

Unhardened Gelatin	4-24	6
Surfactant TX-165 <sup>TM</sup>	0.005-0.1	0.01
HEPES Buffer	2-10	2.4
Ascorbic Acid Oxidase	50,000-300,000 IU/m <sup>2</sup>	175,000 IU/m <sup>2</sup>
Arylacylamidase	1,000-50,000 IU/m <sup>2</sup>	5,000 IU/m <sup>2</sup>

Reagent Layer 2

Hardened Gelatin	4-24	6
Coupler THQSO <sub>3</sub> H	0.2-2	1.0
HEPES Buffer	2-10	4.8
Surfactant TX-165 <sup>TM</sup>	0.005-0.1	0.01

5 KEY

\* g/m<sup>2</sup> except for ascorbic acid oxidase and arylacylamidase which are IU/m<sup>2</sup>.

TX-405<sup>TM</sup>:

TX-100<sup>TM</sup>: A family of octylphenoxy polyethoxy

TX-165<sup>TM</sup>: Ethanol sold by Union Carbide

10 Triton X-100<sup>TM</sup>:

THQSO<sub>3</sub>H: Compound 2 of Table 1, i.e., 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline

Estane: A polyester/polyurethane polymer sold by B. F. Goodrich

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Table 1b

Acetaminophen Element Using Ferricyanide

Coverage, g/m<sup>2</sup>\*

<u>Reagents/Spreading Layer</u>	<u>Useful Range</u>	<u>Actual Example</u>
Barium Sulfate	75-120	100
Cellulose	1-20	8
Surfactant TX-405 <sup>TM</sup>	0.2-4	1.6
Estane	1-15	12.3
Maleimide	0.1-3.0	1.0

Subbing Layer

Polyvinylpyrrolidone	0.5-2.5	1.0
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Reagent Layer 1

Unhardened Gelatin	4-24	6
Surfactant TX-165 <sup>TM</sup>	0.005-0.1	0.01
HEPES Buffer	2-10	2.4
Potassium Ferricyanide	0.1-5.0	1.8
Arylacylamidase	1,000-50,000 IU/m <sup>2</sup>	5,000 IU/m <sup>2</sup>

Reagent Layer 2

Hardened Gelatin	4-24	6
Coupler THQSO <sub>3</sub> H	0.2-2	1.0
HEPES Buffer	2-10	4.8
Surfactant TX-165 <sup>TM</sup>	0.005-0.1	0.01

5 KEY

\* g/m<sup>2</sup> except for arylacylamidase which is IU/m<sup>2</sup>.

TX-405<sup>TM</sup>:

TX-100<sup>TM</sup>: A family of octylphenoxy polyethoxy

TX-165<sup>TM</sup>: Ethanol sold by Union Carbide

10 Triton X-100<sup>TM</sup>:

THQSO<sub>3</sub>H: Compound 2 of Table 1, i.e., 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline

Estane: A polyester/polyurethane polymer sold by B. F. Goodrich

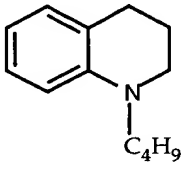
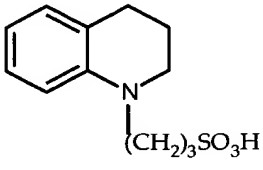
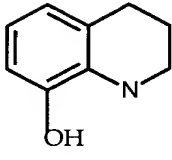
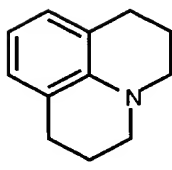
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

### Testing for Useful Coupling Agents

5 The following procedure was used to identify coupling agents which couple with p-aminophenol in the presence of ascorbic acid oxidase to give a strong color compound in solution. Such coupling agents were further tested for their performance in a dry analytical element for quantitatively assaying acetaminophen.

10 Three mL of a 0.1 M TRIS buffer at pH 7.5 was mixed with 300  $\mu$ L of a solution containing 0.04 M of the coupling agent to be tested. To this solution was added 150  $\mu$ L of ascorbic acid oxidase solution (4000 U/mL), 150  $\mu$ L of arylacylamidase solution (70 U/mL), and 150  $\mu$ L of an acetaminophen solution (300 mg/L). After 5 minutes absorbance was measured at 37°C at a wavelength observed for the  $\lambda_{\text{max}}$  for each dye. A spectrum of each dye  
15 solution was made between  $\lambda=300$  and 800 nm in order to determine the  $\lambda_{\text{max}}$  for each dye. Coupling agents which possessed an absorbance greater than 0.3 after 5 minutes were considered satisfactory for use in a dry analytical element (the 0.3 value was determined by subtracting the value of the coupling agent at the wavelength of the  $\lambda_{\text{max}}$  of the dye). The  
20 absorbance values at 300 mg/L acetaminophen for a representative number of coupling agents are listed in Table II. The following nine compounds were among those tested as potential coupling agents:

Table II  
Selection of coupling Agents for Acetaminophen Assay

	<u>Coupling Agent</u>	$\lambda_{\text{max}}$	<u>Absorbance</u>
5	1.		
		670 nm	0.36 (milky solution)
10	2.		
		670 nm	0.65
15	3.		
		600 nm	0.10
20	4.		
		670 nm	0.2

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Table II - Cont'd.  
Selection of coupling Agents for Acetaminophen Assay

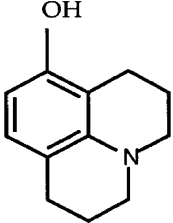
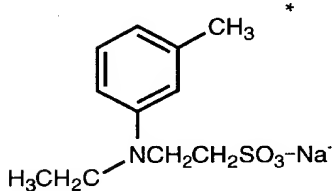
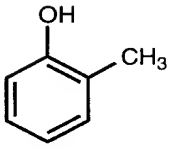
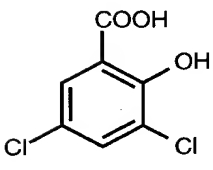
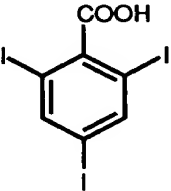
	<u>Coupling Agent</u>	$\lambda_{\text{max}}$	<u>Absorbance</u>
5	5.		
		500 nm	0.23
10	6		
		650 nm	0.12
15	7.		
		600 nm	0.14
20	8.		
		550 nm	0.06
25			

Table II - Cont'd.  
Selection of coupling Agents for Acetaminophen Assay

<u>Coupling Agent</u>	<u><math>\lambda_{\text{max}}</math></u>	<u>Absorbance</u>
5 9. 	650 nm	0.04

10 \* Compounds are water-insoluble. A DMF solution of the compound used was added to the analytical solution.

15 The data of Table II show that, of the coupling agents presented, only Compounds 1, 2, and 4 meet absorbance criteria and therefore were worth testing for use in a dry analytical element (i.e.,  $\lambda = 670$ , absorbance greater than 0.3), with compound 2 producing the highest insensitivity. Compounds 1 and 4 were not soluble without the addition of dimethylformamide (DMF).  
 20 When these compounds were dissolved in a coupler solvent (2,4-di-n-pentylphenol) and then coated in a dry element of the invention (Examples 1, 2, and 3 below), they produced significantly less signal than compound #2. Results are shown in Figures 1, 2, and 3.

Preparation of 1-(3-sulfopropyl)-1,2,3,4-tetrahydroquinoline (compound #2, THQSO<sub>3</sub>H)

25 A mixture of 1,2,3,4-tetrahydroquinoline (4.76 g, 40 mmole) and 1,3-propane sultone (4.88 g, 40 mmole) in 50 mL reagent grade acetonitrile was heated under a nitrogen atmosphere and refluxed overnight. After cooling to room temperature, then to ice bath temperature, the resulting white solid was  
 30 filtered and washed with cold acetonitrile. The yield was 8.1 g (84%), and the product had a melting point of greater than 200°C. Nuclear magnetic resonance (NMR) and elemental analysis showed the product to be consistent with the assigned structure. NMR (D<sub>2</sub>O): 7.45 (s, 4H), 2.8-4.0 (m, 8H), 2.2 (T, 2H).

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Example 1: Coupling Agent THQSO<sub>3</sub>H (No. 2) in Analytical Element

After preparation, coupling compound #2 was coated in the element of the invention shown in Table 1a and tested for its performance in assaying  
5 varying known concentrations of acetaminophen in water (1.0, 5.4, 10.0, 19.0, 30.0, 39.0, 52.0, 98.0, 202.0, and 294.0 mg/L). Coating of the element can be carried out according to the teachings of US Patents 3,992,158, 4,357,363, and 4,258,001.

10 Kinetic response shown in Figure 1 indicates that with this coupler good sensitivity is seen throughout the entire projected dynamic range (1 to 30 mg/dL).

Comparative example 2: Coupling Agent 1-(n-butyl)1,2,3,4-tetrahydroquinoline (compound #1, BTHQ) in Analytical Element

15 Analytical elements were prepared and spotted with samples of varying concentrations of acetaminophen as in Example 1 except that coupling compound #1 (BTHQ) was used in place of compound #2 (THQSO<sub>3</sub>H). Also, 20 because compound No. 1 is not water-soluble, it had to be dispersed in 2,4-di-n-pentylphenol.

Results shown on Figure 2 indicate that this coupling agent is not useful in the analytical element and provides a very narrow dynamic range.

25 Comparative example: Coupling Agent 1,2,3,5,6,7-hexahydro-benzo[i,j]quinolizine, (compound #4, Julolidine) in Analytical Element

30 Analytical elements were prepared and spotted with samples of varying concentrations of acetaminophen as in Example 1 except that coupling compound #4 (Julolidine) was used in place of compound #2 (THQSO<sub>3</sub>H). Because compound No. 4 is not water-soluble, it had to be dispersed in 2,4-di-n-pentylphenol.

35 Results shown on Figure 3 indicate that this coupling agent provides a very narrow dynamic range and so is not useful for our purposes.

Example 2: Tyrosinase as Oxidizing Agent in Element

5 This example was carried out as in Example 1 except that 6,400 U/m<sup>2</sup> of another oxidizing agent, tyrosinase, was used in place of 175,000 U/m<sup>2</sup> of ascorbic acid oxidase. Density response was measured using reflectance densitometry at 670 nm. Predictions were made by deriving a density/prediction spline for the densities produced and predicting the acetaminophen level of the sample from its density.

10 Predictions made based on the density response of the element (Table III) correlates well with the known acetaminophen concentration in the test fluids spotted on the element for testing.

Example Using Tyrosinase as Oxidizing Agent in Element

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Table III

Acetaminophen Level (mg/dL)	Density Response at 670 nm	Prediction (mg/dL)
0.1	.165	0.1
0.5	.178	0.5
1.0	.190	0.9
1.9	.220	1.9
3.0	.251	3.0
3.9	.280	4.1
5.2	.307	5.2
9.8	.495	10.0
20.9	.650	20.8
29.4	.723	29.4

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Example 3: Using Potassium Ferricyanide as Oxidizing Agent in Element

20 After preparation, coupling compound #2 was coated in the element of the invention using potassium ferricyanide as the oxidizing agent as shown in Table 1b. It was tested for its performance in assaying varying known concentrations of acetaminophen in human serum (0.0, 10, 24, 50, 100, 146, 193, 247, 295, 350, and 391 mg/L).

- Kinetic response shown in Figure 4 indicates that the ferricyanide provides good sensitivity throughout the entire projected dynamic range (1 to 30 mg/dL). Use of other metal salts, (Table A) in the element structure of Table 1b were too slow. It was unexpected that the ferricyanide would produce faster kinetics based on its lower redox potential when compared to the other oxidizing agents trialed.
- 5

Table A

<u>Material</u>	<u>Redox Potential</u>	<u>Reaction Rate</u>
Cupric Sulfate	+0.34V	Slow
Cupric Chloride	+0.34V	Slow
Ferric Nitrate	+0.77V	Very Slow
Silver Nitrate	+0.59V	Very Slow
Silver Nitrate	+0.81V	Very Slow
Potassium Ferricyanide	+0.46V	Very Fast

The invention has been described in detail with particular reference to a preferred embodiment thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.